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Protective role for collectin-11 in rheumatoid arthritis in mice

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Disclosure

There are no other potential conflicts of interest relevant to this article.

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Abstract

Objective. Collectin-11 (CL-11) is a soluble C-type lectin, a mediator of innate immunity. Its role in autoimmune disorders is unknown. The goal of this study was to determine the role of CL-11 in a mouse model of rheumatoid arthritis (RA).

Methods. A murine collagen-induced arthritis (CIA) model, combining both gene deletion of *Colec11* and recombinant (rCL-11) treatment approaches were employed. Joint inflammation and tissue destruction, circulating levels of inflammatory cytokines and adaptive immune responses were assessed in CIA mice. Splenic $CD11c^+$ cells were used to examine the influence of CL-11 on antigen presenting cell (APC) function. Serum levels of CL-11 in RA patients were also examined.

Results. *Colec11*$^{-/-}$ mice developed more severe arthritis than WT mice (as determined by disease incidence, clinical arthritis scores and histopathology; $P<0.05$). Disease severity is associated with significantly enhanced APC activation, Th1/Th17 responses, pathogenic IgG2a production and joint inflammation, as well as elevated circulating levels of inflammatory cytokines. In vitro analysis of $CD11c^+$ cells revealed that CL-11 is critical for suppression of APC activation and function. Pharmacological treatment of mice with rCL-11 reduced the severity of CIA in mice. Analysis of human blood samples revealed that serum levels of CL-11 was lower in RA patients (n=51) compared to healthy controls (n=53), a serum CL-11 reduction also displays a negative relationship with DAS28, ESR and CRP ($P<0.05$).

Conclusion. Our findings demonstrate a novel role for CL-11 in protection against RA, suggesting the underlying mechanism involved suppression of APC activation and subsequent T cell responses.

Introduction

Collectins are a group of soluble C-type lectins; mannose-binding lectin (MBL), collectin-10 and lung surfactant proteins (SP-A, SP-D) are well-known members among the group. They function as pattern recognition receptors (PRRs) that bind to carbohydrates or carbohydrate moieties on the surface of pathogens and host cells, they play roles in modulation of cellular processes in addition to participating in host defense (1-3).

Collectin-11 (CL-11, also known as CL-K1) is another member of the collectin family displaying structural similarities with other collectins. However, it displays some unique characteristics such as having a wide tissue distribution, a relatively low serum concentration, and binding a wide range of ligands (4-7). These suggest CL-11 may be involved in a broad range of cellular processes, and local production of CL-11 may play important roles in these cellular processes. CL-11 is highly conserved among species; human and mice show 92% homology at the amino acid level (5). Functional protein is derived from the *COLEC11* gene on chromosome 2 in humans and 12 in mice. CL-11 has been shown to play important roles in embryogenesis and host defense and mediate the pathogenesis of renal ischemia reperfusion injury (8-11). Additionally, in a recent study in retinal pigment epithelial cells (RPE), we found that CL-11 upregulates IL-10, but downregulates IL-6 production by the cells, suggesting a critical role of CL-11 in immune regulation (12). Together, these studies suggest that CL-11 is a multifunctional molecule that can participate in diverse biological processes. To date, the functional roles of CL-11 have been described in either non-immune or innate immune settings. It is unknown whether CL-11 plays an important role in adaptive immunity.

Rheumatoid arthritis (RA) is a chronic, progressive inflammatory autoimmune disease, manifesting both joint and systemic effects. It is characterized by general synovial inflammation, cartilage destruction and bone erosions, with accompanying elevation of circulating autoantibodies and systemic inflammation (13). The pathogenesis of RA is not fully understood, but it is thought that genetic susceptibility and environmental factors (e.g. smoking, infections) trigger abnormal autoimmune responses, which involve both innate and adaptive immune responses (14). Previous studies have suggested that the pathogenic

process in RA involves several stages. These include: i) the induction of adaptive immune responses that lead to T cell and B cell activation and autoantibody production (the initial phase), ii) subsequently, the occurrence of synovial inflammation, with inflammatory cell infiltration, cell activation, and increase in cytokine production (the effector phase), and iii) eventually, the inflammation is converted to a chronic process which leads to the release of cytokines, proteases, and other mediators that cause tissue destruction (the chronic phase) (15).

The dysregulation of adaptive immune responses is thought to play a significant role in the pathogenesis of RA (13). Clinical studies have shown that multiple autoantibodies - due to epitope spreading- can be detected in the serum and synovium of RA patients (16), and T cell co-stimulation blockade produces significant clinical and functional benefits in patients who have had an inadequate response to anti-TNF-α therapy (17). Experimental studies in murine models of RA have further supported the importance of $CD4^+$ T cells, particularly Th17 cells in the pathogenesis of RA, particularly in the initial phase of the autoimmune reaction and in inducing local inflammation in the joints (18). Despite the major role of the adaptive immune response, innate immune responses could also play important roles in the initiation of RA. Adaptive immunity effector mechanisms, such as the above-mentioned action of Th17 cells, requires the participation of innate cells and cytokine/chemokines. The inflammatory environment plays a key role in shaping adaptive immune responses. Indeed, a large number of studies have shown that innate immune responses play important roles in RA. Proinflammatory cytokine/chemokine production, inflammatory cell infiltration, and complement activation in synovium have been implicated in tissue inflammation and bone destruction (19-21). However, most of these studies have focused on the role of innate immune responses in the pathogenesis of effector and chronic phases. Little is known about whether/how innate immune system can act in concert with adaptive immune responses and impinge on the initiation of RA.

Given CL-11 is a pattern recognition molecule with multiple potential functions that also possesses immune regulatory properties, we hypothesized that CL-11 might play an important role in shaping adaptive immunity, there by influencing the development of RA. In

the present study, we investigated this hypothesis. A murine model of RA induced by collagen, combining both deletion of *Colec11* gene and recombinant (rCL-11) treatment approaches were used to determine the role of CL-11 in disease development and progression. Joint inflammation and tissue destruction, circulating levels of inflammatory cytokines and adaptive immune responses were assessed following collagen immunization. Splenic $CD11c^+$ cells were used to examine the influence of CL-11 on antigen presenting cell (APC) cytokine secretion and function in T cell stimulation. Additionally, we assessed the clinical relevance of CL-11 in RA by measuring serum levels of CL-11 in RA patients.

Materials and Methods

Mice: Homozygous *Colec11*$^{-/-}$ mice on a C57BL/6 background (22) were purchased from Mutant Mouse Resource and Research Centers (UC Davis, Davis, CA) and have been back-crossed onto the C57BL/6 strain for least 8 generations. WT littermates (WT) were used as controls. Male mice (10-14 weeks) were used in all experiments unless specified. All mice were maintained in specific pathogen-free conditions on a 12-hour reversed light/dark cycle. The Ethics Review Committee for Animal Experimentation at Xi'an Jiaotong University approved and oversaw all mouse experiments.

Patients: A total of 51 patients diagnosed with rheumatoid arthritis and 53 healthy donors were enrolled. The study was approved by the Ethics Committee Board of the No. 5 Hospital of Xi'an and the Second Affiliated Hospital of Xi'an Jiaotong University. Informed consent was obtained from each participant according to the regulations of our institutional ethics committee. The clinical characteristics of the patients are provided in Supplementary Table 1.

Induction of collagen-induced arthritis (CIA) and clinical evaluation: CIA was induced in WT and *Colec11*$^{-/-}$ mice or rCL-11-treated mice using a previously described protocol for the induction of arthritis in C57BL/6 mice (23) (sFig. 1A). Clinical arthritis was evaluated using a previously described scoring system (24, 25), the incidence of arthritic paws was defined as the occurrence of inflamed paws with a clinical arthritis score of 2 or more (24) by two independent observers in a blinded manner. Serum levels of cytokine,

C3a/C5a and collagen-specific IgG/IgG2a were measured by ELISA. Histopathological changes were assessed using hematoxylin and eosin (H&E) and toluidine blue (T-blue)-stained-sections by two independent observers in a blinded manner. Cellular infiltration in joint tissue was assessed by immunohistochemistry. Tissue inflammation was assessed by RT-PCR for key inflammatory mediators. Splenocytes isolated from WT CIA and *Colec11*$^{-/-}$ CIA mice were used for evaluating frequencies of $CD4^+$, $CD8^+$ T and B cells and for detection of IFN-γ or IL-17A producing cells by flow cytometry analysis.

In vitro experiments: Splenic $CD11c^+$ cells were prepared from WT and *Colec11*$^{-/-}$ mice and used to investigate the impact of CL-11 on APC cytokine secretion and capacity to stimulate T cells by flow cytometry and ELISA.

Statistical analyses: Data are shown as mean ± SD. Unpaired t-test was used to compare the means of two groups. Paired t-test was used to compare the means of matched-pairs. ANOVA was used to compare the means of more than two independent groups. Pearson's correlation coefficient was used to measure the degree of relationship between the two groups of data. All analyses were performed using Graphpad Prism 7 software. Two tailed $P < 0.05$ was considered significant.

Additional materials and methods: Reagents and more detailed methods for clinical arthritis scores, histopathology of knee joints, immunohistochemistry, detection of CL-11, CD11c and CD4 in spleen, analysis of T cell and B cell responses in CIA mice ex vivo, isolation of $CD11c^+$ cells and $CD4^+$ T cells, analysis of anti-specific T cell responses in vitro, flow cytometry analysis, RT-qPCR, and ELISA are described in the Supplementary Materials and Methods.

Results

Colec11$^{-/-}$ mice develop more severe CIA

We induced CIA in *Colec11*$^{-/-}$ and WT mice to determine the role of CL-11 in this model of RA. The incidence and severity of arthritis were assessed visually from 24 days after the first immunization (3 days after the second boost immunization), at intervals of 2 days, up to 42 days. *Colec11*$^{-/-}$ mice displayed a higher incidence of arthritis than WT mice (Figure 1A). *Colec11*$^{-/-}$ mice also developed more severe arthritis with significantly higher clinical scores than WT mice across all time points (Figure 1B and 1C). H&E and T-blue staining showed that *Colec11*$^{-/-}$ CIA mice exhibited more severe synovial inflammation (i.e. synovial edema and hyperplasia, inflammatory infiltrates in synovium, pannus formation and its invasion of articular cartilage and bone), cartilage damage (loss of proteoglycan on the surface), bone erosion, compared with WT CIA mice (Figure 1D-1E). Immunohistochemistry showed the number of macrophages (F4/80^{+}) and neutrophil (Ly6B.2^{+}) in knee joint tissue was higher in *Colec11*$^{-/-}$ CIA mice compared to WT CIA mice (Figure 1F). Together, these results demonstrate that CL-11 deficiency promotes more severe arthritis.

Colec11$^{-/-}$ CIA mice display enhanced joint inflammation and elevated circulating levels of inflammatory cytokines

Joint inflammation was analyzed by examining tissue mRNA levels of key proinflammatory cytokine by RT-qPCR. *Colec11*$^{-/-}$ CIA mice had significantly higher mRNA levels of *Tnf-*α*, Il-1*β*, Il-6, Ccl8, Ccl2* and *Rankl* compared with WT mice (Figure 2A). Circulating levels of inflammatory cytokines in CIA mice were analyzed by ELISA. *Colec11*$^{-/-}$ CIA mice had significantly higher serum levels of TNF-α, IL-6 and CCL2, but lower levels of IL-10 compared with WT mice (Figure 2B). These results demonstrate that CL-11 deficiency leads to enhanced joint inflammation and systemic inflammatory responses. There was no significant difference in basal levels of inflammatory cytokines between naïve *Colec11*$^{-/-}$ mice and WT mice (sFig. 2A), indicating that differences observed in cytokine production in WT CIA and *Colec11*$^{-/-}$ CIA mice resulted from differential inflammatory responses to collagen antigen.

Colec11$^{-/-}$ mice have enhanced adaptive immune responses following collagen immunization

Adaptive immune responses contribute to the pathogenesis of RA in humans and CIA in mice. We therefore assessed the impact of CL-11 deficiency on adaptive immune responses following collagen immunization. We examined serum levels of T helper cell index cytokines and collagen-specific antibodies in WT CIA and *Colec11*$^{-/-}$ CIA mice on day 42 post-induction. *Colec11*$^{-/-}$ CIA mice had significantly higher serum levels of IFN-γ and IL-17A as well as collagen-specific total IgG and IgG2a than WT CIA mice (Figure 2B and 2C). These results indicate that CL-11 deficiency leads to enhanced adaptive immune responses following antigen immunization.

CL-11 deficiency-dependent enhancement of adaptive immune responses was further evaluated by analyzing T cell and B cell activation in WT CIA and *Colec11*$^{-/-}$ CIA mice on day 25 and day 42 from isolated splenocytes. *Colec11*$^{-/-}$ CIA mice had significantly higher frequencies of splenic CD4$^+$ T cells and CD8$^+$ T cells on day 25 compared with WT CIA mice. However, the frequency of CD19$^+$ B cells was even lower in *Colec11*$^{-/-}$ CIA mice (potentially reflecting a relative change in the CD45$^+$ compartment) (Figure 2D). Frequencies of regulatory T cells in the spleen were comparable between the WT and *Colec11*$^{-/-}$ CIA mice (sFig. 3). Next, we analyzed IFN-γ and IL-17A-producing CD4$^+$ T cells in splenocytes isolated from WT CIA and *Colec11*$^{-/-}$ CIA mice. Compared to WT CIA mice, *Colec11*$^{-/-}$ CIA mice had a significantly higher percentage of IFN-γ$^+$ and IL-17A$^+$ cells in CD4$^+$ T cell population on both day 25 and day 42 (Figure 2E). There was no statistically significant difference in the basal levels of T cell and B cell activities as well as serum cytokines between naïve *Colec11*$^{-/-}$ mice and WT mice (sFig. 2B and 2C).

APC maturation/activation is critically important for the initiation and direction of T cell responses. We therefore investigated whether CL-11 deficiency can impact APC maturation/activation. We analyzed CD11c$^+$ cells from the draining lymph nodes (LNCD11c$^+$) of WT and *Colec11*$^{-/-}$ mice 24h after collagen immunization. LNCD11c$^+$ cells of *Colec11*$^{-/-}$ mice displayed higher levels of surface MHC class II, CD86 and CD40 than the cells of WT mice (Figure 2F).

Together, these results indicate that ablation of CL-11 leads to enhanced adaptive immune responses following the collagen immunization.

Impact of CL-11 deficiency on APC function

Impact of CL-11 deficiency on APC function was evaluated using both splenic $CD11c^+$ ($SPCD11c^+$) cell culture and a co-culture model for measuring antigen-specific T cell responses. Isolated $SPCD11c^+$ cells (prepared from WT and *Colec11*$^{-/-}$ mice) were further cultured for 24h, and inflammatory cytokine secretion was measured. Compared to $SPCD11c^+$ cells from WT mice, $SPCD11c^+$ cells from *Colec11*$^{-/-}$ mice produced higher levels of proinflammatory cytokines (i.e. TNF-α, IL-12), but lower level of IL-10 (Figure 3A). Isolated $SPCD11c^+$ cells (prepared from WT and *Colec11*$^{-/-}$ mice) were also co-cultured with syngeneic splenic $CD4^+$ T cells (prepared from collagen antigen primed C57BL/6) in the presence of collagen antigen for up to 72h, and T cell responses were analyzed by measuring IFN-γ or IL-17A producing $CD4^+$ T cells and cytokine secretion. Compared to $SPCD11c^+$ cells from WT mice, $SPCD11c^+$ cells from *Colec11*$^{-/-}$ mice induced higher T cell responses, as evidenced by increased IFN-γ producing and IL-17A producing $CD4^+$ T cells (Figure 3B). In addition, higher levels of IL-2, IFN-γ, IL-17A, IL-12 and TNF-α were observed in co-culture supernatants (Figure 3C, 3D). These results, together with in vivo observations made in $LNCD11c^+$ cells indicate that CL-11 deficiency leads to enhanced APC activation and function.

Next, we assessed the possibility of an interaction of CL-11 with APCs and T cells in the spleen. Immunochemical staining for CL-11, CD11c and CD4 was performed on spleen sections (24h after the boost immunization), to examine the distribution and localization of CL-11. CL-11 was clearly detected in the spleen, which was mainly distributed in the marginal zone and T cell zone and less in the germinal center. A majority of the $CD11c^+$ cells were positively stained for CL-11, $CD4^+$ T cells rarely positively stained for CL-11 (Figure 3E). Thus, abundant distribution of CL-11 in the marginal zone and T cell zone, and colonization of CL-11 with $CD11c^+$ cells support an interaction of CL-11 with APCs and possibly T cells.

Pharmacological treatment of mice with rCL-11

Having demonstrated the role of CL-11 in protection against CIA by using *Colec11*$^{-/-}$ mice, we next explored whether pharmacological treatment of mice with rCL-11 could reduce CIA. Two treatment protocols were employed, including early treatment (starting 24h before the induction of CIA) and late treatment (starting on the day of the boost immunization) (sFig. 1B, 1C). Severity of arthritis, serum levels of proinflammatory cytokines and antigen specific IgG levels were assessed as described in figure 1. Compared with the control group, mice receiving early treatment with rCL-11 developed less severe CIA, as evidenced by reduced arthritis scores, reduced serum levels of IFN-γ, IL-17A, TNF-α and collagen II-specific IgG/IgG2a. In contrast, serum levels of IL-10 were higher in the rCL-11 treatment group than that of the control group (Figure 4A-4C). Mice receiving late treatment with rCL-11 also displayed reduced arthritis scores and collagen II-specific IgG2a levels, (but higher IL-10 levels), when compared to the control group, though the differences were small. Other pathological parameters including serum cytokines (TNF-α, IFN-γ, IL-17A) and collagen II-specific total IgG levels were not statistically different between the two groups of mice, although there was a trend toward a reduction in the rCL-11-treated group (Figure 4D-4F). These results indicate that pharmacological treatment of mice with rCL-11 reduces CIA; with the early treatment protocol providing improved protection when compared to the late treatment protocol.

Clinical relevance of CL-11 in RA patients

We explored the clinical relevance of CL-11 in RA patients. We analyzed the serum levels of CL-11 in RA patients (n=51) and healthy controls (n=53) by ELISA. The CL-11 levels in RA patients were significantly lower compared with the controls (Figure. 5A). We also performed correlation analysis in RA patient samples to assess the strength of the relationship between CL-11 levels and DAS28 or the levels of several inflammatory and

immunological molecules that are usually present in a person who has RA. CL-11 levels were negatively correlated with DAS28, erythrocyte sedimentation rate (ESR) and C-reactive protein levels (CRP) (Figure 5B). There was no apparent correlation between CL-11 levels and either anti-cyclic citrullinated peptide antibody (anti-CCP) or rheumatoid factor (RF) levels (Figure 5C). These observations indicate a negative relationship between the serum levels of CL-11 and the severity of RA as well as key inflammatory markers (ESR, CRP).

Discussion

Although it is well recognized that both innate and adaptive immune responses contribute to the pathogenesis of autoimmune diseases such as RA, there is relatively less known about the regulation at the interface of the two arms of the immune system, particularly in the early phase of the disease. In this study, we demonstrate a novel role for CL-11 as an important link between innate and adaptive immune responses, where by CL-11 mediates suppression of APC activation/function and subsequent T cell responses, ultimately limiting inflammatory responses, thereby conferring protection against CIA.

We employed the murine CIA model in mice absent of the *Colec11* gene or via pharmacologic administration of rCL-11 in WT mice, to determine the role of CL-11 in CIA. The results of our *in vivo* experiments, by measuring multiple parameters (i.e. clinical arthritis scores, joint tissue inflammation/destruction, serum levels of proinflammatory cytokines and antigen-specific antibodies), clearly show that CL-11 is required for suppression of CIA, as mice lacking CL-11 developed more severe CIA. These observations support a protective role for CL-11 in the development and progression of destructive arthritis.

In addition to demonstrating the protective role of CL-11 in CIA, we investigated how CL-11 confers protection observed against CIA. A key finding in this regard is that *Colec11*$^{-/-}$ mice exhibited markedly enhanced Th1 and Th17 responses and antigen-specific antibody production. This suggests that CL-11 has a suppressive effect upon adaptive immune responses. To elucidate potential mechanisms of CL-11-dependent suppression of the adaptive immune response, we identified CL-11 as critical for suppression of APC

activation/function. This conclusion was supported by several findings from *in vivo* and *ex vivo* analyses of APCs. The first finding was that $CD11c^+$ cells from draining lymph nodes of immunized *Colec11*$^{-/-}$ mice displayed a more activated phenotype, with enhanced expression of MHC class II and co-stimulatory molecules. The second observation was that $SPCD11c^+$ cells from *Colec11*$^{-/-}$ mice secreted more proinflammatory cytokines. Additionally, $SPCD11c^+$ cells from *Colec11*$^{-/-}$ mice, when co-cultured with syngeneic $CD4^+$ T cells in the presence of collagen antigen, induced enhanced antigen specific T cell responses.

An important question arising from our observation upon the impact of CL-11 deficiency on $CD11c^+$ cell activation is how absence of CL-11 leads to overactivation of $CD11c^+$ cells. One possibility is that extracellular CL-11 released from dendritic cells (DC) and possible bystander cells, as well as from the circulation, could establish suppressive effects on cell activation/maturation. This could be supported by our two observations. Firstly, in the present study, we found that abundant distribution of CL-11 in the marginal zone and T cell zone, and colocalization of CL-11 with $CD11c^+$ cells. Secondly, in a separate study, we showed that $BMCD11c^+$ cells can synthesize and secrete CL-11, and the addition of rCL-11 to $BMCD11c^+$ cell culture medium inhibited proinflammatory cytokine production in these cells (26). It is known that CL-11 has a wide spectrum of carbohydrate ligands. Notably, CL-11 has been shown to bind mannose residues on retinal epithelial cells and fibroblasts, thereby modulating cell function (11, 12). Accordingly, it is conceivable that CL-11 can modulate DC function (in an autocrine or paracrine manner) through engagement of carbohydrate ligand on the cell surface. Apart from the extracellular effect, CL-11 may exert a suppressive effect on DC through other mechanisms such as intracellular actions, which warrants further investigation. Therefore, ablation of CL-11 could result in loss of suppressive effects on DC, thereby leading to cellular overactivation.

Based on our findings and previously published observations, we propose that CL-11 is an important negative regulator of APC activation (Figure 6). CL-11 deficiency causes APC overactivation, leading to enhanced T cell responses (particularly Th1/Th17), which may (directly or indirectly) promote systemic and local inflammatory responses, antigen-specific antibody production, thus contributing to CIA. In contrast, with CL-11 sufficiency, CL-11

mediates suppression of APC activation and subsequent T cell responses, resulting in minimal inflammation and tissue damage.

Another important finding in this study is that early administration of rCL-11 reduces the severity of CIA in mice. Given the amino acid sequence of CL-11 is highly conserved between human and mouse, the efficacy of rCL-11 in protection against CIA in mice may have therapeutic implications for human RA (e.g. RA at preclinical stage or in first degree relatives with anti-CCP positivity). The mechanisms by which administration of rCL-11 mediates protection are unclear. There are several potential mechanisms. First, rCL-11 could modulate the activation/function of APCs (e.g. monocytes) both in the circulation and lymphoid tissues as rCL-11 can penetrate lymphoid tissues (sFig. 4). Second, rCL-11 may mediate direct effects on T cell activation. Third, rCL-11 may mediate anti-inflammatory effects on innate immune cells. Administration of rCL-11 after the onset of disease also achieved a certain degree of protection against CIA, which may reflect the involvement of a direct inhibitory effect of CL-11 on T cell activation and/or CL-11-mediated anti-inflammatory effects on monocyte/macrophage.

Our findings in the preclinical CIA model of RA and the availability of a specific and sensitive ELISA for human CL-11 (27) motivated us to test the clinical relevance of CL-11 in RA patients. By measuring serum levels of CL-11 in RA patients and healthy controls and by performing correlation analyses, we determined that serum levels of CL-11 were associated inversely with DAS28 and key inflammatory markers (ESR, CRP). The mechanisms involved in the presence of low levels of CL-11 in RA are unknown, but could result from dysregulated synthesis of CL-11, hyper-consumption, or genetic variations existing in the general population (28), which warrants further investigation. Our findings are consistent with previous observations that patients with SLE have significantly lower serum levels of CL-11 compared with normal controls (29), suggesting an association between lower CL-11 serum levels and autoimmune disorders.

In view of the spectrum of biological functions of collectins, studies in surfactant proteins and MBL have suggested that binding of collectins to carbohydrate ligands on the surface of pathogens or host cells, not only leads to activation of the lectin pathway, but also mediates

regulation of multiple cellular processes (30, 31). This also appears to be true for CL-11. Studies in 3MC syndrome patients, murine models of renal ischemia reperfusion injury and lung *S. pneumoniae* infection suggest the role of CL-11 in these pathologies is dependent upon complement activation. However, complement activation-independent functions of CL-11, such as opsonophagocytosis of apoptotic cells and cell proliferation, have also been reported in previous studies (11, 12). Furthermore, in this study no significant differences in circulating levels of C3a/C5a and synovial C3d deposition between the WT CIA and *Colec11*$^{-/-}$ CIA mice (sFigs. 5 and 6), suggesting that the protective role for CL-11 observed in this model was achieved independent of complement activation. Overall, these studies support the notion that CL-11 as a multifunctional molecule participates in pathophysiology via different mechanisms, in a complement activation-dependent or -independent manner.

The limitations of this study include: i) being focused on the mechanism of CL-11-mediated suppression of APC activation/function, does not exclude the possibility that CL-11 may also provide protection against CIA by influencing other immune cell functions (e.g. T cells, macrophages and regulatory T cells; ii) our study does not address how ablation of CL-11 causes overactivation of APC, which warrants further studies; iii) as a paradigm shift in immune response regulation may exist in CIA, proinflammatory cytokines, especially for IFN-γ, may change to having anti-inflammatory effects. Our study therefore does not exclude the possibility that upregulation of IFN-γ in *Colec11*$^{-/-}$ CIA mice might also exert anti-inflammatory effects in this model; iv) the sample size for the RA patient study was too small, and therefore the results may not be completely generalizable.

In conclusion, our findings reveal a novel role for CL-11 in protection against CIA in mice, suggesting that CL-11 confers protection through suppression of both APC activation and subsequent Th1 and Th17 responses. In addition, our data show that CL-11 plays an important role in limiting the development of RA and should be explored as a possible future treatment option.

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Figure legends

Figure 1. *Colec11*$^{-/-}$ mice develop more severe CIA

(**A**) The incidence of arthritic paws, (**B**) Clinical arthritis scores from d24 to d42, and (**C**) clinical arthritis scores on d42, in WT and *Colec11*$^{-/-}$ mice [X^2 test in **A**, Two-way ANOVA with multiple comparisons test in **B** and Unpaired t-test in **C,** n=40 (WT) and 36 (*Colec11*$^{-/-}$) mice/group]. (**D**) Representative microscopic images of H&E and T-blue staining in knee joint sections on d42. Arrows indicate lesions and abnormalities in the joint. Scale bars: 200 μm. (**E**) Separate histological scores (Two-way ANOVA with multiple comparisons test, n=8 mice/group). (**F**) Representative images of immunohistochemistry for F4/80 (macrophage) and Ly6B.2 (neutrophil) in knee joint sections of WT CIA and *Colec11*$^{-/-}$ CIA mice on d42 (n=3 mice/group). Arrows indicate the positively stained cells. Scale bars: 20 mm. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001. All quantitative data were presented as Mean±SD.

Figure 2. *Colec11*$^{-/-}$ mice have enhanced innate and adaptive immune responses following collagen immunisation

(**A**) Relative mRNA levels of proinflammatory mediators in hind paw tissues (Unpaired t-test, n=8 mice/group). (**B**) Serum cytokine levels and (**C**) serum collagen II-specific IgG and IgG2a levels (Unpaired t-test, n=22 in WT CIA mice, n=18 in *Colec11*$^{-/-}$ CIA mice). (**D**) Frequencies of $CD4^+$, $CD8^+$ and $CD19^+$ cells in the spleen, and (**E**) Percentage of IFN-γ^+ and IL-$17A^+$ cells in $CD4^+$ T cell population of WT CIA and *Colec11*$^{-/-}$ CIA mice (d25, d42), determined by flow cytometry (Unpaired t-test , n=6 mice per group). Lower panel: Representative flow cytometry graphs. (**F**) Flow cytometry analysis for surface expression of MHC class II (MHC II), CD86, and CD40 in $LNCD11c^+$ cells of WT and *Colec11*$^{-/-}$ mice 24h after collagen immunisation [Unpaired t-test (n=10 mice/group). Lower panel: Representative flow cytometry graphs. All quantitative data were presented as Mean±SD. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001. The dotted line across the graph represents the expression levels of normal mice, which is similar between WT and *Colec11*$^{-/-}$ mice.

Figure 3. Impact of CL-11 deficiency on function

(**A**) Cytokine levels in 24h culture supernatants of SPCD11c$^+$ cells derived from WT or *Colec11*$^{-/-}$ mice in the presence of LPS (10 ng/ml) (by ELISA). (**B**) Percentage of IFN-γ^+ and IL-17A$^+$ cells in CD4$^+$ T cell population within the co-culture of primed CD4$^+$ T cells and SPCD11c$^+$ cells derived from WT or *Colec11*$^{-/-}$ mice in the presence of collagen for 72 h (by Intracellular staining/flow cytometry analysis). (**C, D**) Cytokine levels in 48h or 72h co-culture supernatants in **B** (by ELISA). (**E**) Immunochemical staining for CL-11 (red), CD11c (green), CD4 (white) and nuclei (DAPI, blue) was performed on the spleen section of mouse 24h after the boost immunization. Top panel: showing that positive staining for CL-11 was detected in the marginal zone (MZ) and T cell zone (TZ) and less in germinal center (GC). Scale bar: 50 μm. Lower panel: high magnification images corresponding to the boxed regions in the top panel showing majority CD11c$^+$ cells were positively staining for CL-11 in the MZ (indicated by the arrows) (image 1), only rare CD4$^+$ T cells were positive stained for CL-11 in the TZ (image 2). Scale bars: 10 μm. (**A-D**) Data were analysed by paired t-test (n=3-4 independent experiments). *, P<0.05; **, P<0.01; ***, P<0.001.

Figure 4. Pharmacological treatment of mice with rCL-11

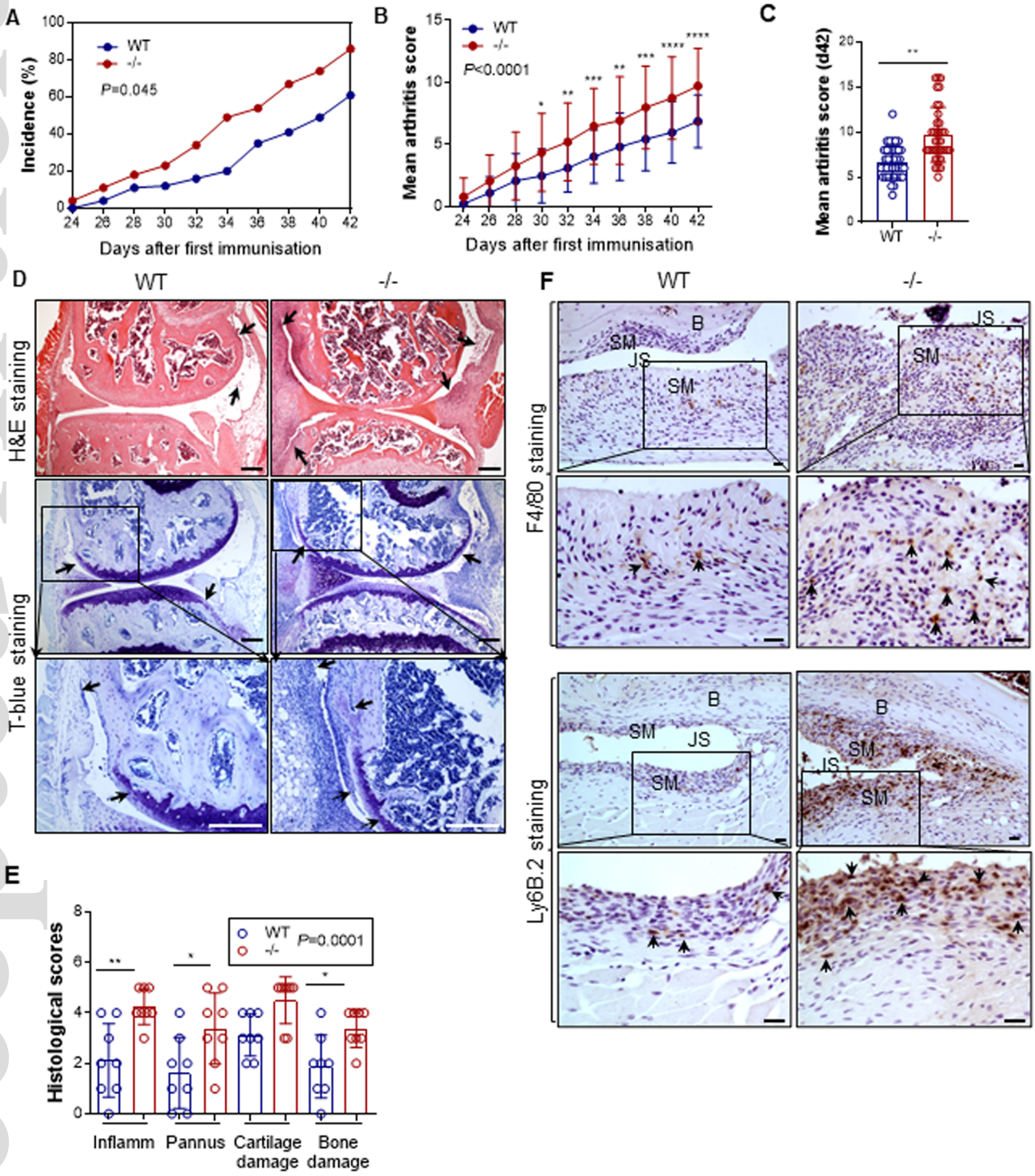
(**A-C**) Administration of rCL-11 or BSA (Ctrl) to WT mice started 24h before the 1st immunisation. (**A**) Clinical arthritis scores from d24 to d42 (Two-way ANOVA with multiple comparisons test, n=10 mice/group]). (**B**) Serum cytokine levels and (**C**) serum collagen II-specific total IgG/IgG2a levels (Unpaired t-test, n=10 mice/group). (**D-F**) Administration of rCL-11 or BSA (Ctrl) to WT mice started d21 after the 1st immunisation. (**D**) Clinical arthritis scores from d24 to d42 (Two-way ANOVA with multiple comparisons test, n=12 mice/group]). (**E**) Serum cytokine levels and (**F**) serum collagen II-specific total IgG/IgG2a levels (Unpaired t-test, n=12 mice/group). The dotted line across the graph represents each cytokine levels of normal mice. *, P<0.05; **, P<0.01; ***, P<0.001; n.s., no significant. All quantitative data were presented as Mean±SD.

Figure 5. Serum levels of CL-11 in patients with RA and its association with disease activity

(**A**) Serum levels of CL-11 in patients with RA and heathy controls, determined by ELISA. Data were analysed by Unpaired two-tailed t-test (n=51 in RA patients, n=53 in the controls). (**B, C**) The association between CL-11 and disease activity scores (DAS28), ESR, CRP, anti-CCP or RF. Data were analysed using Pearson's correlation.

Figure 6. Proposed mechanism for the protective role of CL-11 in CIA

Schematic diagram of proposed mechanism for the protective role of CL-11 in CIA. Based on our findings in this study, we propose that CL-11 deficiency causes DC overactivation, leading to enhanced T cell responses (particularly Th1/Th17), which may promote systemic and local inflammatory responses, thus contributing to collagen-induced arthritis. In contrast, with CL-11 sufficiency, CL-11 mediates suppression of DC activation and subsequent T cell responses, resulting in minimal inflammation and tissue damage.



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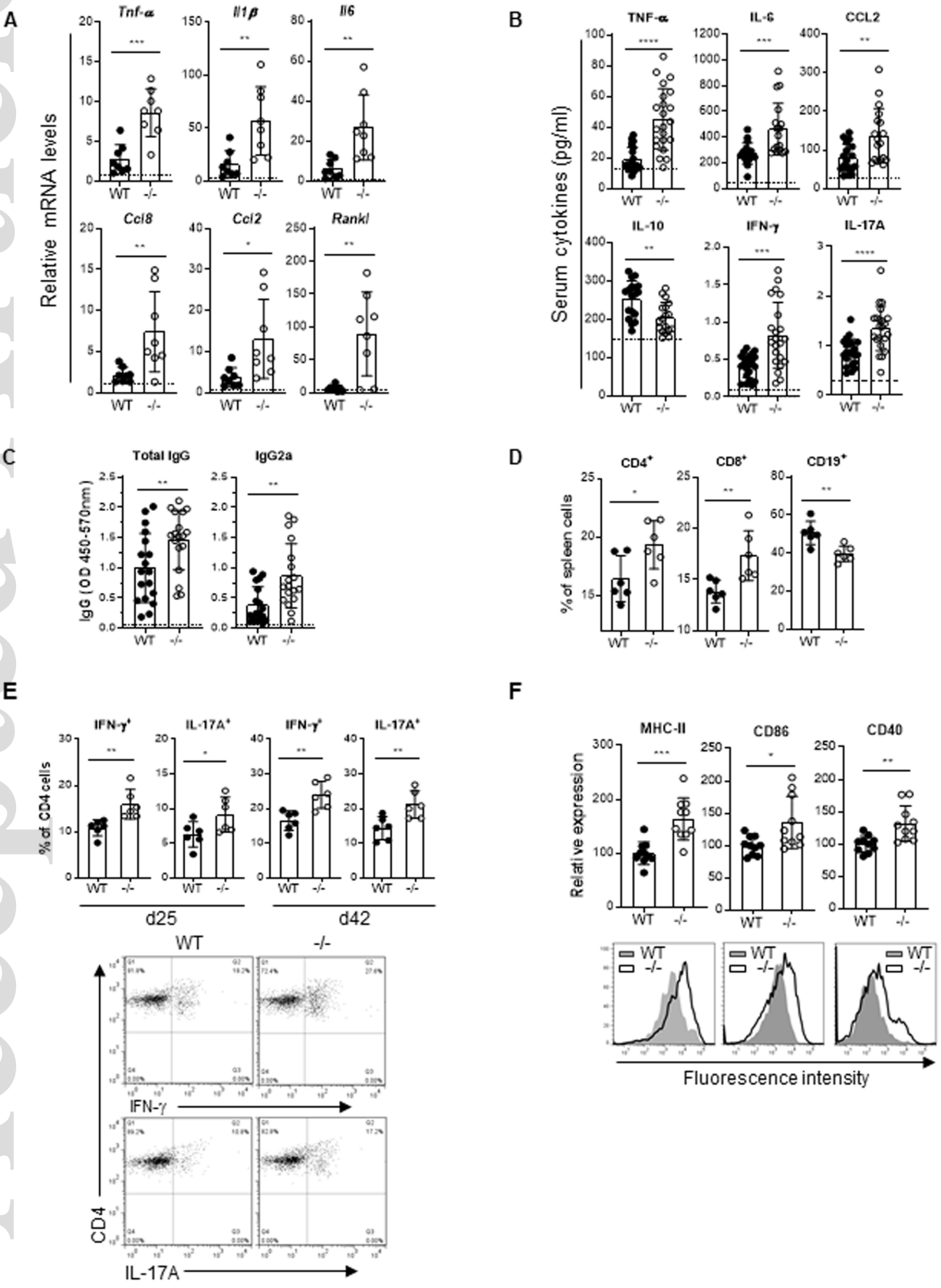

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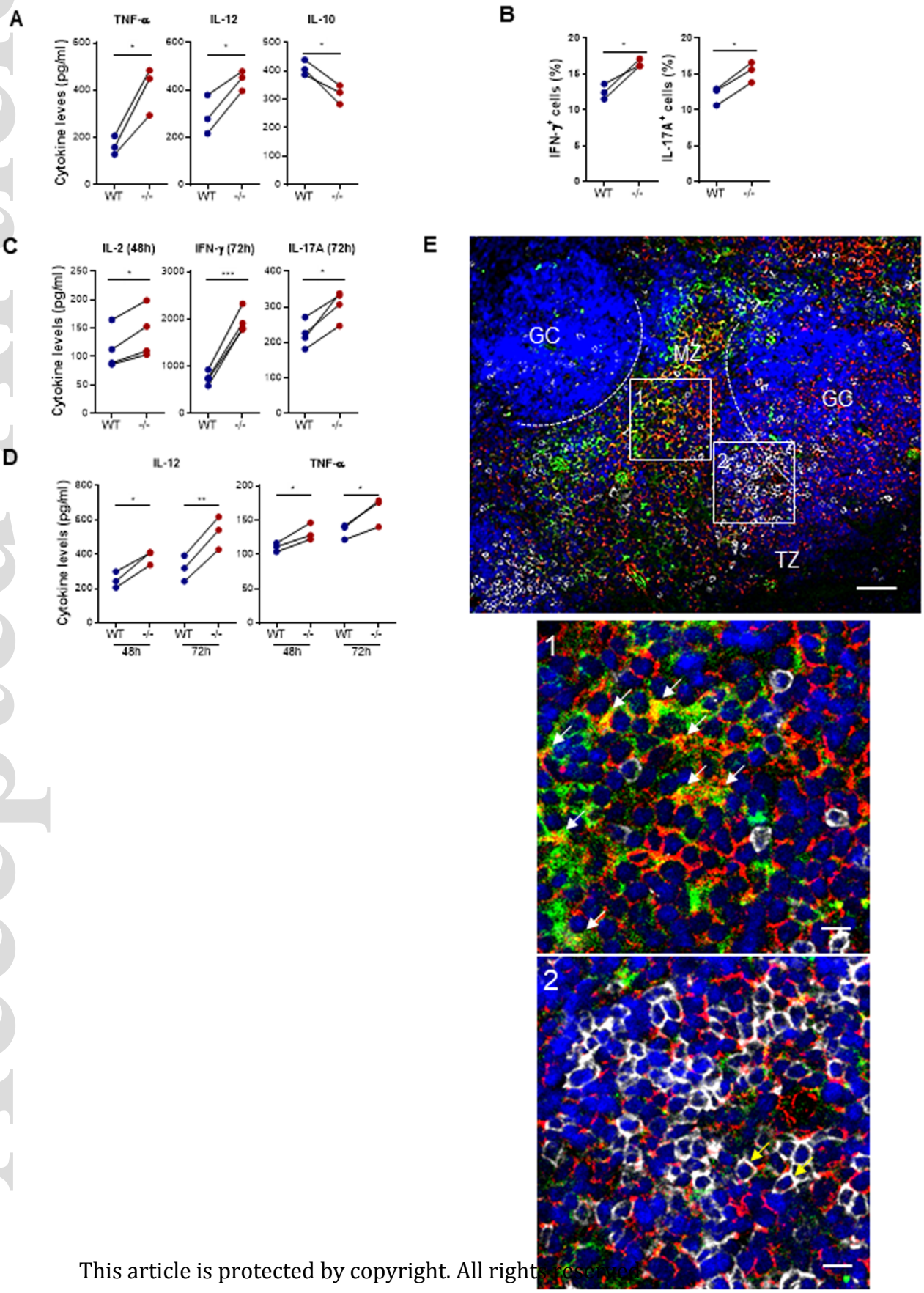

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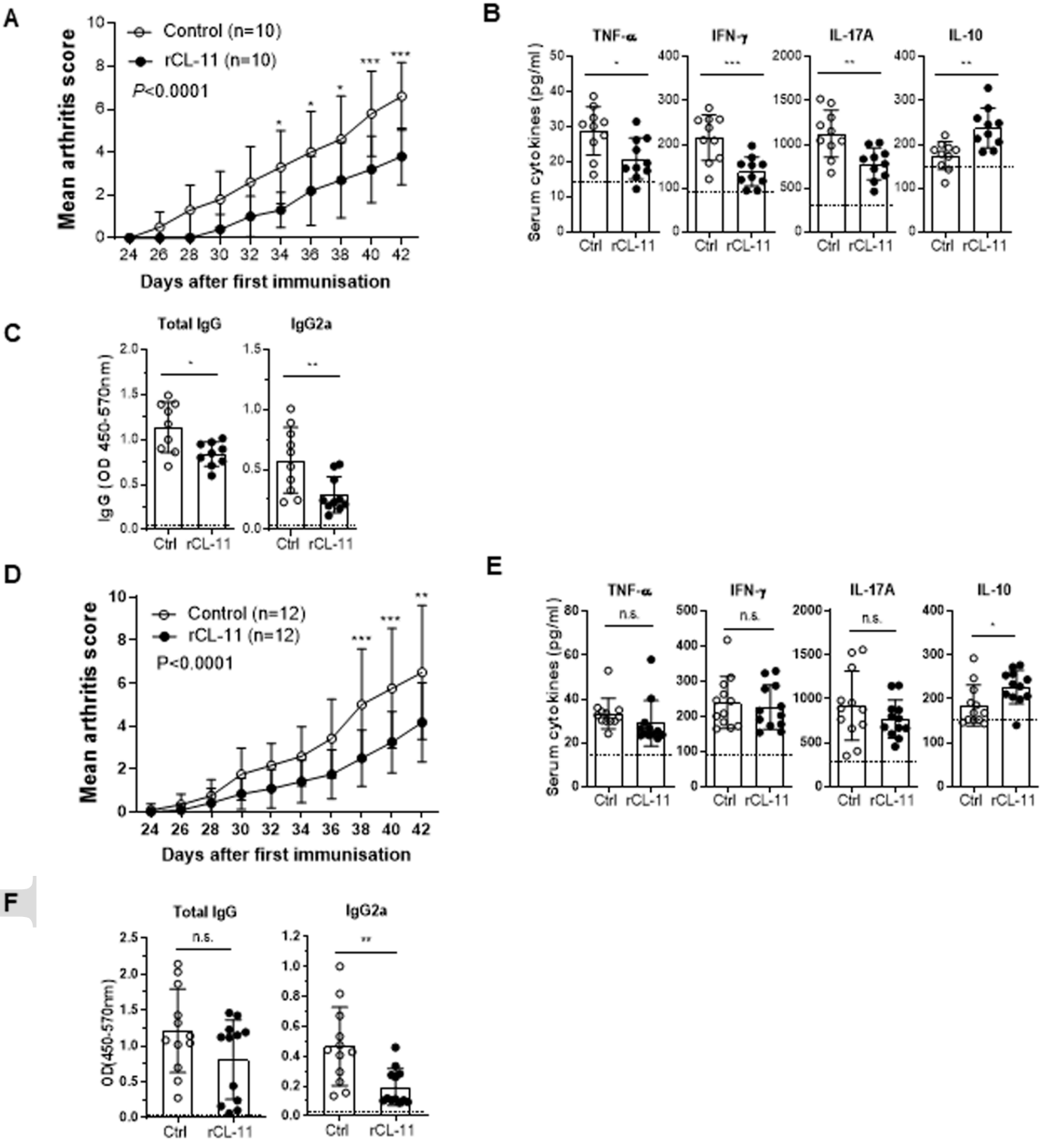

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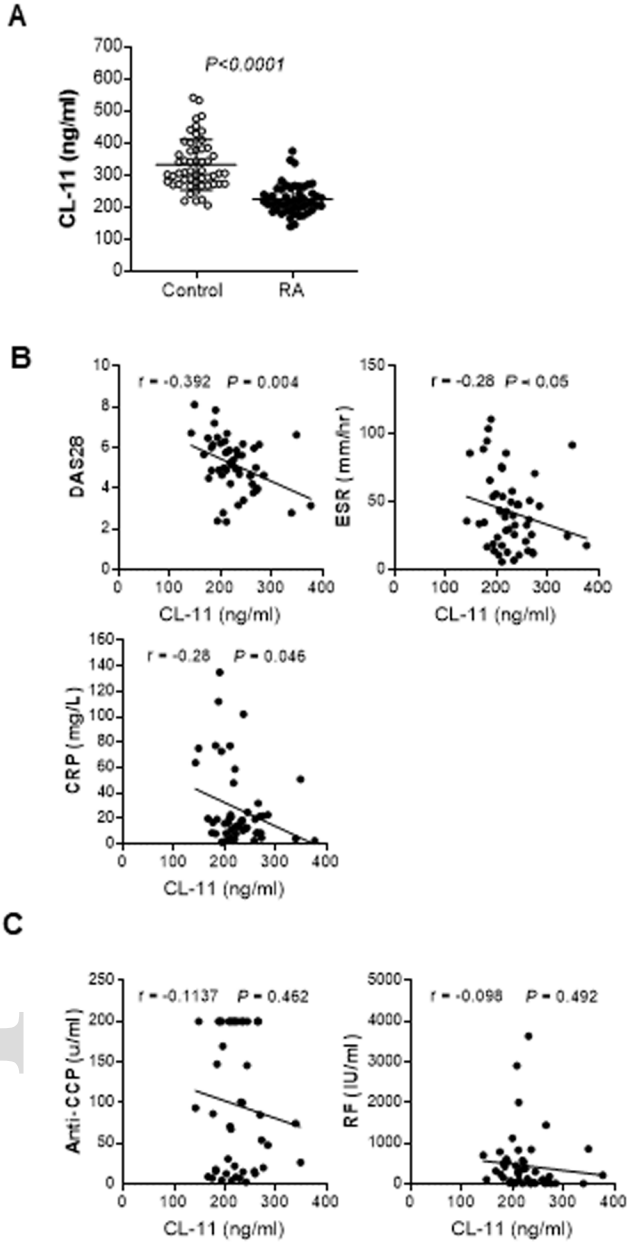

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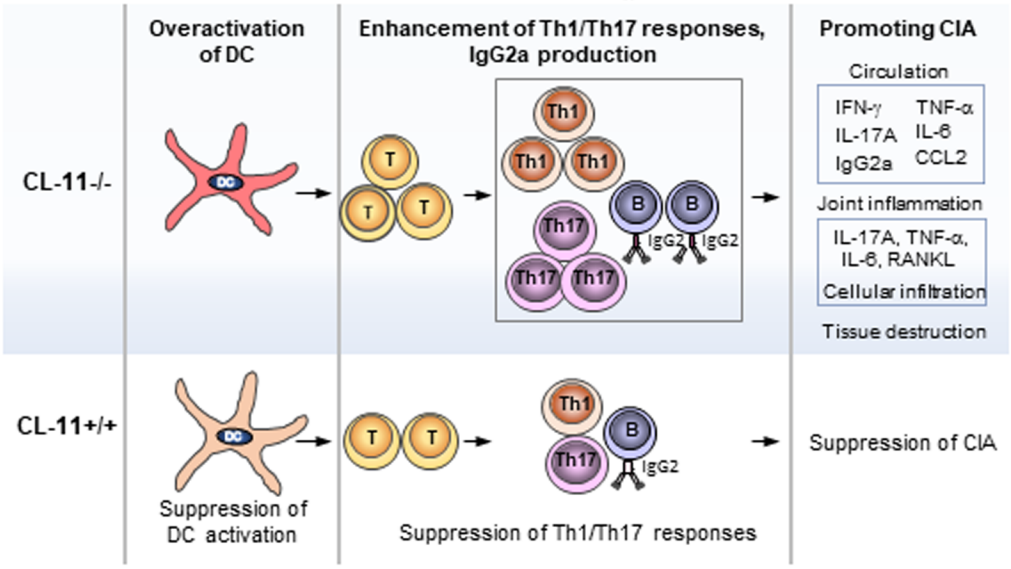

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